



Whole-exome sequencing identifies an α -globin cluster triplication resulting in increased clinical severity of β -thalassemia

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Abstract Whole-exome sequencing (WES) has been increasingly useful for the diagnosis of patients with rare causes of anemia, particularly when there is an atypical clinical presentation or targeted genotyping approaches are inconclusive. Here, we describe a 20-yr-old man with a lifelong moderate-to-severe anemia with accompanying splenomegaly who lacked a definitive diagnosis. After a thorough clinical workup and targeted genetic sequencing, we identified a paternally inherited β -globin mutation (*HBB*:c.93-21G>A, *IVS-I-110*:G>A), a known cause of β -thalassemia minor. As this mutation alone was inconsistent with the severity of the anemia, we performed WES. Although we could not identify any relevant pathogenic single-nucleotide variants (SNVs) or small indels, copy-number variant (CNV) analyses revealed a likely triplication of the entire α -globin cluster, which was subsequently confirmed by multiplex ligation-dependent probe amplification. Treatment and follow-up was redefined according to the diagnosis of β -thalassemia intermedia resulting from a single β -thalassemia mutation in combination with an α -globin cluster triplication. Thus, we describe a case where the typical WES-based analysis of SNVs and small indels was unrevealing, but WES-based CNV analysis resulted in a definitive diagnosis that informed clinical decision-making. More generally, this case illustrates the value of performing CNV analysis when WES is otherwise unable to elucidate a clear genetic diagnosis.

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INTRODUCTION

Whole-exome sequencing (WES) has been remarkably successful at helping clinicians and researchers identify pathogenic single-nucleotide variants (SNVs) or small indels that result in severe disease (Sankaran et al. 2012, 2015; Sankaran and Gallagher 2013; Yang et al.

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2013; Lacy et al. 2016). In a smaller number of cases, rare copy-number variants (CNVs) that are inferred from differences in WES read coverage have also been identified as pathogenic variants (de Ligt et al. 2013; Poultney et al. 2013; Kelsen et al. 2015; Miyatake et al. 2015). Typically, however, putative CNV calls are not a component of the clinical sequencing deliverables. Indeed, WES-based CNV calls are generally of lower quality than whole-genome sequencing (WGS), microarray, or array comparative genomic hybridization-based calls (Samarakoon et al. 2014; Belkadi et al. 2015), although these approaches have additional cost when compared with WES-based CNV calling when WES data are already available.

β -Thalassemia intermedia is typically caused by mutations that affect copies of the β -globin gene that limit, but do not completely abrogate, the production of functional β -globin chains, resulting in a moderate anemia, often with iron overload and other comorbidities (for review, see Vichinsky 2016). The presence of a heterozygous β -globin mutation concurrent with a triplication of the α -globin gene have been described in a number of patients with a clinical presentation ranging from thalassemia minor to intermedia (Kanavakis et al. 1983; Sampietro et al. 1983; Galanello et al. 1983; Thein et al. 1984; Henni et al. 1985; Camaschella et al. 1987; Kulozik et al. 1987; Oron et al. 1994; Traeger-Synodinos et al. 1996; Bianco et al. 1997; Giordano et al. 2009; Farashi et al. 2015; Mehta et al. 2015; Clark et al. 2016). In most cases, the genetic changes were $\alpha\alpha^{\text{anti}3.7}$, the counterpart of the $\alpha^{-3.7}$ deletion, the most common deletion of the α -globin locus, which spans 3.7 kb and involves both the $\alpha 2$ - and $\alpha 1$ -globin genes. This genetic change is easily detectable by restriction endonuclease mapping or multiplex Gap-PCR (Liu et al. 2000; de Mare et al. 2010). Here, we performed WES and searched for pathogenic variants in a 20-yr-old man who was initially clinically diagnosed with a rare congenital form of anemia (congenital dyserythropoietic anemia or CDA). Although we could not identify any pathogenic SNVs or indels in the known CDA genes, WES-based CNV analysis revealed an α -globin triplication that was coinherited with a heterozygous β -globin mutation, resulting in a definitive diagnosis of β -thalassemia intermedia that impacted the ongoing clinical management of this patient.

RESULTS

Clinical Presentation

At the age of 18, the patient was first referred to our hematology clinic at the Schneider Children's Medical Center of Israel for evaluation of a lifelong anemia. Medical records showed that at 8 mo of age hemoglobin of 7 g/dl (normal: 10.5–14 g/dl) was noted, while MCV was 62 fl (normal: 73–85 fl). Reticulocyte count was 4%, LDH and indirect bilirubin were elevated, and iron status and thyroid function tests were normal. The father was of Eastern-European origin and the mother was an Ashkenazi Jew. The parents were non-consanguineous. Upon presentation to our clinic, a physical examination was performed. The patient appeared jaundiced and his spleen was palpated 12 cm below the costal margin. The patient also developed cholelithiasis. Hemoglobin levels ranged between 7 and 9 g/dl, whereas the MCV ranged between 62 and 70 fl. A bone marrow aspiration revealed erythroid hyperplasia with mild dyserythropoiesis, a few binucleated erythroid precursors, and some megaloblastic changes (Fig. 1). Iron staining revealed no evidence of sideroblastic anemia. A thorough workup was performed to rule out enzymopathies, including glucose 6-phosphate dehydrogenase, pyruvate kinase, phosphofructokinase, glucosephosphate isomerase, phosphoglycerate kinase, and aldolase deficiency. Membrane defects were ruled out by the osmotic fragility test.

A molecular workup revealed that the patient was a carrier of the *HBB*:c.93-21G>A (IVS-I-110:G>A) mutation in the β -globin gene (Young et al. 1985). The father was found to

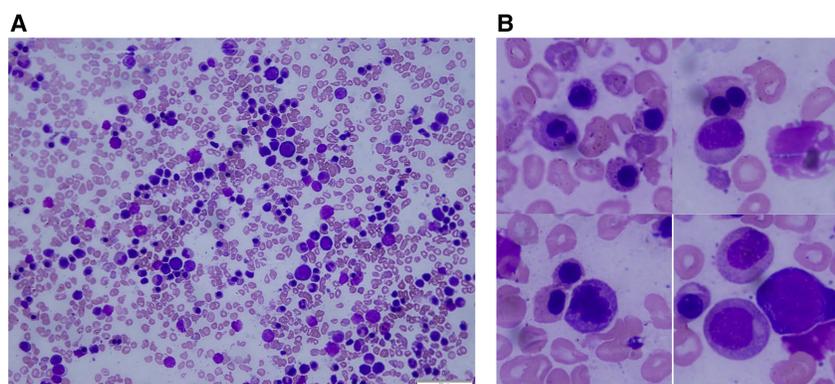


Figure 1. Bone marrow morphology of the patient demonstrating erythroid hyperplasia (A) and dyserythropoietic changes (B). Scale bar, 50 μ M.

carry the same mutation, although he had not previously come to clinical attention for this mild case of β -thalassemia minor. Therefore, the presence of one copy of the mutated allele was unlikely to be sufficient to cause the severe lifelong anemia and morphological changes observed and additional genetic causes were investigated. Sequencing and multiplex gap-PCR of the α -globin gene did not reveal any abnormalities, including the $\alpha^{-3.7}$ deletion and $\alpha\alpha^{\text{anti}3.7}$ triplication (Liu et al. 2000; de Mare et al. 2010). Given the mild dyserythropoiesis, the CDA genes (*CDAN1*, *C15ORF41*, *SEC23B*, *KIF23*, *KLF1*, and *GATA1*) were sequenced but no pathogenic variants could be identified.

Genomic Analyses

Given the unrevealing results of the targeted genetic approaches, whole-exome sequencing was performed on the patient (Supplemental Table 1). Forty-six candidate erythroid genes were first investigated for rare (ExAC AF < 0.01%) and potentially damaging (loss-of-function or missense) mutations, but no candidate variants for the anemia could be identified. Given that our typical WES-based SNV and small indel analysis was also unrevealing, we decided to call putative CNVs using WES read coverage. This analysis resulted in the identification of eight putative CNVs (Table 1) that were present at a frequency of <5% in control samples. Inspection of these rare putative CNVs revealed a possible triplication of the α -globin cluster (Chr16:160473-240621), in addition to the known mutation in the β -globin gene (Fig. 2). Although maternal DNA was not available to determine the exact inheritance pattern,

Table 1. Rare copy number variants identified in proband

Chromosome	Start	End	Event	No. of exons
1	92,251,714	92,433,879	Duplication	7
2	109,271,481	109,287,355	Deletion	4
5	177,171,344	177,210,906	Duplication	7
6	610,047	656,996	Deletion	7
7	100,606,688	100,610,365	Deletion	7
11	89,664,289	89,715,334	Duplication	9
16	160,473	240,621	Duplication	23
17	19,532,927	19,536,653	Deletion	4

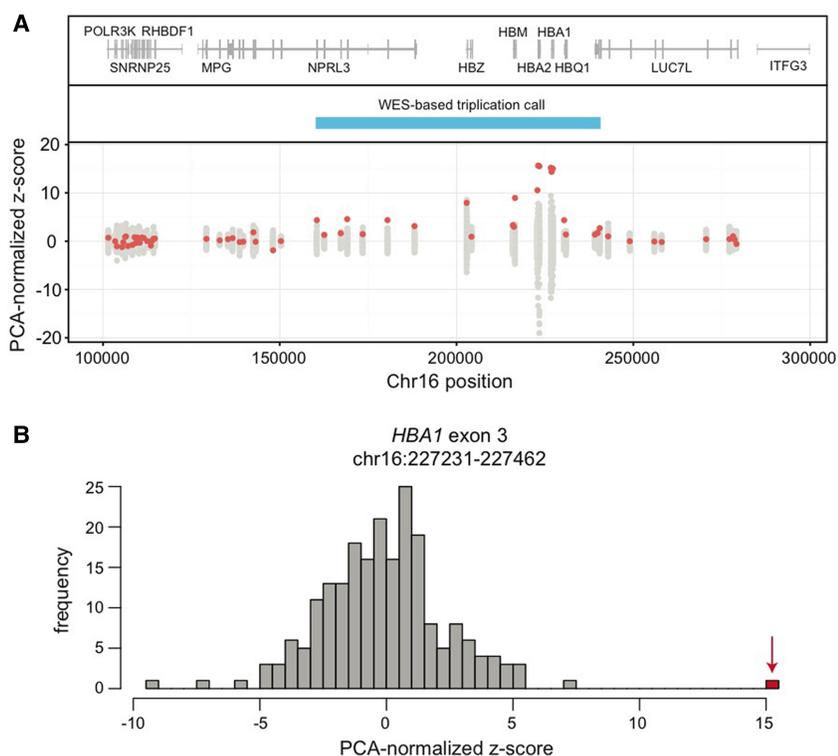


Figure 2. Coverage differences in whole-exome sequencing revealed the possibility of an α -globin locus triplication. (A) Plot of principal component analysis (PCA)-normalized z-scores of mean centered read coverages across α -globin locus for 216 controls (gray) and case (red). Deviation from the control distribution of these scores indicates the presence of a likely deletion or duplication. Multimapped reads were allowed given the high homology between *HBA1* and *HBA2* in order to estimate the full length of the CNV event. (B) Plot of PCA-normalized z-scores for specific bait targeting *HBA1* exon 3.

multiplex ligation-dependent probe amplification (MLPA) confirmed that the patient harbored a large triplication of the whole α -globin cluster including *HBA1*, *HBA2*, and *HS40-HBQ1-3* (Fig. 3). No other complete genes were present in this triplication event. Importantly, this triplication was larger than would be detected with typical multiplex gap-PCR approaches.

Treatment Outcomes

Thalassemia results from imbalance in the levels of α - and β -globin chains. In β -thalassemia, the α/β globin ratio is increased, resulting in excess α -globin, which is toxic to red blood cell precursors. Generally, β -thalassemia symptoms can be partially ameliorated by mutations that reduce the overall synthesis of α -globin but worsened by mutations that result in excess α -globin.

The combination of α -thalassemia triplications and heterozygous β -thalassemia mutations are known to result in a clinical presentation of thalassemia intermedia (Galanello et al. 1983; Thein et al. 1984; Henni et al. 1985; Camaschella et al. 1987; Kulozik et al. 1987; Oron et al. 1994; Traeger-Synodinos et al. 1996; Giordano et al. 2009; Farashi et al. 2015; Mehta et al. 2015; Clark et al. 2016). In this patient, the diagnosis of thalassemia intermedia is in agreement with his clinical workup, including microcytic anemia and splenomegaly. Having determined the correct diagnosis of thalassemia intermedia in our patient helped guide his clinical management, including decisions regarding indications for

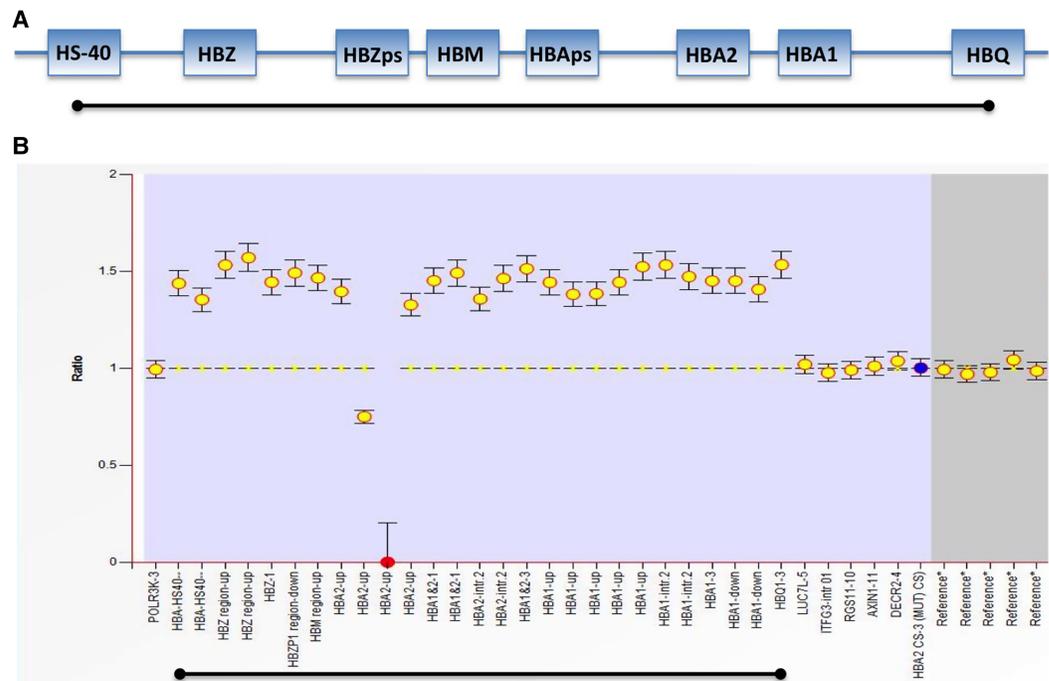


Figure 3. MLPA verifies a triplication of the whole α -globin cluster. (A) A schematic presentation of the α -globin locus including the genes involved in the triplication by MLPA studies. The black bar indicates the extent of the triplication. (B) MLPA is a multiplex PCR method for detecting abnormal copy numbers. The y-axis represents the relative quantity of each amplicon. The black bar indicates the area of the triplication as diagnosed by MLPA. The error bars refer to the standard deviation. The triplication begins upstream of the DNase I hypersensitive site HS-40 (an upstream regulatory element of *HBA* cluster genes) and ends downstream to the *HBQ1* gene. *The reference genes (gray background) are located in other areas of the genome and serve as a validation of the technique in each run.

blood transfusion, monitoring and treating complications of chronic hemolysis and of iron overload, periodic assessment for pulmonary hypertension, and consideration for treatment with agents that induce fetal hemoglobin (Taher et al. 2013), as the increased production of γ -globin improves the imbalance of the α - and β -globin chains (Musallam et al. 2013). Most importantly, as a result of a definitive molecular diagnosis, we decided to not perform a surgical splenectomy, which can ameliorate the severity of certain anemias (Lacy et al. 2016) but instead carries a high risk of postsplenectomy complications in thalassemia intermedia patients (Karimi et al. 2011; Taher et al. 2013).

DISCUSSION

The clinical spectrum resulting from the combination of heterozygosity for β -thalassemia and triplication of α -globin is wide and ranges from mild β -thalassemia minor to clinically significant thalassemia intermedia (Kanavakis et al. 1983; Sampietro et al. 1983, Galanello et al. 1983, Thein et al. 1984; Henni et al. 1985, Camaschella et al. 1987; Kulozik et al. 1987; Oron et al. 1994; Traeger-Synodinos et al. 1996; Bianco et al. 1997; Giordano et al. 2009; Farashi et al. 2015; Mehta et al. 2015). Most commonly, the triplication of α -globin results from abnormal homologous recombination, generating the common $\alpha^{-3.7}$ deletion and the $\alpha\alpha^{\text{anti}3.7}$ triplication. However, larger duplications, such as the one identified here, have been described (Harteveld et al. 2008; Jiang et al. 2015; Clark et al. 2016). These larger

triplications seem to uniformly result in more severe disease, at least as far as has been reported in the literature, likely because they result in the addition of two extra α -globin copies, contrasting with the $\alpha\alpha^{\text{anti3.7}}$ triplication, which adds only one α -globin copy.

Importantly, these uncommon triplications are often missed by standard molecular technologies such as restriction endonuclease mapping and multiplex gap-PCR. Recently, three duplications of the α -globin locus that extended well beyond the locus and would be undetectable by standard approaches were identified by targeted genome sequencing (using a unique library of DNA baits) in thalassemia intermedia patients with heterozygous mutations in the β -globin gene (Clark et al. 2016). Here, we show that in addition to this approach and targeted MLPA (Harteveld et al. 2008; Colosimo et al. 2011), standard WES can also be used to identify extended α -globin CNVs. Thus, in certain cases where WES has already been performed, WES-based CNV calling provides a cost-effective approach for the identification of copy-number changes across the α -globin locus.

Notably, the clinical and laboratory details of this patient initially suggested a diagnosis of CDA, and the patient underwent a full genetic workup for all genes known to be involved in these syndromes. In agreement with our own clinical experience as well as with other recent studies, targeted or exome sequencing of clinical CDA cases occasionally results in no genetic evidence of CDA but definitive evidence of alternative hematological disorders explanative of the CDA-like phenotype (Roy et al. 2016). In these alternative disorders, stress erythropoiesis potentially leads to the CDA-like phenotypes observed, but an accurate diagnosis can often have a significant impact on clinical decision-making in these cases.

WES-based CNV calling has successfully identified pathogenic variants in a number of genetically unsolved cases. Variants identified are typically deletions, but here we show that pathogenic increases in copy number can also be successfully identified in WES. More generally, we suggest that WES-based CNV calling should be a standard part of any WES pipeline and putative CNVs should be carefully investigated when standard variant analyses are unrevealing prior to moving to more expensive approaches such as WGS.

METHODS

Peripheral blood and bone marrow samples were collected after informed consent was obtained. Bone marrow aspiration smears were stained with Hematek (Siemens).

Sequencing and Analysis

DNA was extracted by DNA isolation kit from mammalian blood (Roche) according to the manufacturer's instructions. WES was performed as previously described with the exception that Illumina ICE baits were used for DNA capture (Sankaran et al. 2012). Coverage across the consensus coding DNA sequences (downloaded from UCSC genome browser on December 13, 2015) plus an additional 20 nt on each side of the exons was calculated using Picard tools (Table 1). The variant call file was annotated with Variant Effect Predictor v83 (McLaren et al. 2016). The genome analysis toolkit (GATK) and Bcftools were then used to identify rare variants (DePristo et al. 2011; Li 2011; Lek et al. 2016). No rare (defined as 0.01% allele frequency in ExAC) damaging (missense or loss-of-function) mutations were present in the patient in any of the known CDA or other red cell disorder genes (*ANK1*, *SPTB*, *SPTA1*, *SLC4A1*, *EPB42*, *EPB41*, *PIEZO1*, *KCNN4*, *GLUT1*, *G6PD*, *PKLR*, *NT5C3A*, *HK1*, *GPI*, *PGK1*, *ALDOA*, *TPI1*, *PFKM*, *ALAS2*, *FECH*, *UROS*, *CDAN1*, *SEC23B*, *KIF23*, *KLF1*, *GATA1*, *HBB*, *HBA1*, *HBA2*, *RPS19*, *RPL5*, *RPL11*, *RPL35A*, *RPS26*, *RPS24*, *RPS17*, *RPS7*, *RPS10*, *RPL26*, *RPS29*, *RPS28*, *RPS27*, *RPL27*, *RPL15*, *RPL31*, *TSR2*) with the exception of IVS-I-110:G>A in *HBB*. Copy-number variant analysis was performed using XHMM (Fromer et al. 2012). Controls were 216 unrelated cases from a Diamond–Blackfan anemia

cohort that were sequenced at the Broad Institute at the same time as the case reported here. Suggested XHMM parameters were used with the exception that CNVs were called using both unique and multimapped exon read coverages (GATK DepthOfCoverage parameters “-minMappingQuality 20” and “-minMappingQuality 0”, respectively), because *HBA1* and *HBA2* are highly homologous.

Multiplex Ligation-Dependent Probe Amplification

MLPA was performed using the commercially available kit Salsa MLPA P140B HBA (MCR-Holland) following the manufacturer’s instructions. The amplified fragments were separated by capillary electrophoresis in the 3130XL Genetic Analyzer, ABI PRISM (Applied Biosystems). Quantitative data were obtained with Gene-Mapper v3.7 software (Applied Biosystems).

ADDITIONAL INFORMATION

Data Deposition and Access

Whole-exome sequencing data have been deposited in the dbGaP database (<http://www.ncbi.nlm.nih.gov/gap>) under the accession number phs000474.v3.p2. The HBB variant has been submitted to ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar>), accession number SCV000579457.

Ethics Statement

The study was approved by the Institutional Review Board (IRB) of the Rabin Medical Center (Study number 0031-11). The patient provided a written consent for the genetic analysis. The IRB allows for the genetic testing performed to be analyzed and deposited, as indicated.

Author Contributions

O.S.S., R.L., and H.T. contributed to patient recruitment and phenotyping. J.C.U. and V.G.S. contributed to sequence data analysis and interpretation. O.S.S., J.C.U., S.N.L., T.K., O.D., R.L., V.G.S., and H.T. contributed to functional evaluation of the variant. O.S.S., J.C.U., V.G.S., and H.T. contributed to writing the initial draft of the manuscript. All authors contributed to revising the manuscript and reviewing the final draft.

Competing Interest Statement

The authors have declared no competing interest.

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